

Folate-mediated targeted and intracellular delivery of paclitaxel using a novel deoxycholic acid-*O*-carboxymethylated chitosan–folic acid micelles

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Background: A critical disadvantage for successful chemotherapy with paclitaxel (PTX) is its nontargeting nature to cancer cells. Folic acid has been employed as a targeting ligand of various anticancer agents to increase their cellular uptake within target cells since the folate receptor is overexpressed on the surface of such tumor cells. In this study, a novel biodegradable deoxycholic acid-*O*-carboxymethylated chitosan–folic acid conjugate (DOMC-FA) was used to form micelles for encapsulating the anticancer drug PTX.

Methods and results: The drug-loading efficiency, encapsulation efficiency, in vitro drug release and physicochemical properties of PTX-loaded micelles were investigated in detail. In vitro cell culture studies were carried out in MCF-7 cells, a human breast carcinoma cell line, with folate receptor overexpressed on its surface. An increased level of uptake of folate-conjugated micelles compared to plain micelles in MCF-7 cells was observed, and the enhanced uptake of folate-micelles mainly on account of the effective process of folate receptor-mediated endocytosis. The MTT assay, morphological changes, and apoptosis test implied that the folate-conjugated micelles enhanced the cell death by folate-mediated active internalization, and the cytotoxicity of the FA-micellar PTX (DOMC-FA/PTX) to cancer cells was much higher than micelles without folate (DOMC/PTX) or the commercially available injectable preparation of PTX (Taxol).

Conclusion: Results indicate that the PTX-loaded DOMC-FA micelle is a successful anticancer-targeted drug-delivery system for effective cancer chemotherapy.

Keywords: paclitaxel, folate, polymeric micelles, targeted delivery

Introduction

Paclitaxel (PTX), one of the most successful anticancer drugs, is the first of a new class of microtubule-stabilizing agents, and has demonstrated significant antitumor activity in clinical trials against a broad range of solid tumors, especially against non-small-cell lung cancer, metastatic breast cancer, and refractory ovarian cancer.^{1,2} However, because of low therapeutic index and the poor aqueous solubility of approximately 1 µg/mL of PTX, the generally used commercial preparation of PTX is Taxol (Bristol-Myers Squibb, New York, NY), a concentrated solution containing 6 mg PTX/mL of Cremophor EL (polyoxyl 35 castor oil; Sigma-Aldrich, St Louis, MO) and dehydrated alcohol (1:1, v/v), which is diluted 5–20-fold in normal saline or dextrose solution before administration.¹ Unfortunately, serious side effects have been reported, such as hypersensitivity, neurotoxicity, nephrotoxicity, endothelial and

vascular muscles causing vasodilatation, labored breathing, and lethargy attributable to intravenous administration of the current Cremophor EL-based formulation.³ For this reason the extensive clinical application of this drug is extremely limited.²

Because of these problems, there is a need for the development of an alternate formulation of PTX with good aqueous solubility, targeting delivery to tumor cells, and the ability to reduce side effects. Accordingly, a number of alternative formulations were investigated for solubilization of PTX, including nanoparticles, liposomes, microspheres, PTX-polymer conjugates, dendritic polymers, implants, and water-soluble prodrugs.^{1,2,4} Although these vehicles employed have shown much promise for replacing the Cremophor EL-based vehicle for PTX delivery, approaches of actively targeting delivery of PTX to tumor cells have seldom been considered. Therefore, to improve the therapy effect and decrease the side effects of PTX, delivering PTX by using a targeted drug delivery system is a potential approach.

O-carboxymethylated chitosan (OCMC) is a kind of carboxymethylated derivative of chitosan, with good biocompatibility, bioactivity, antibacterial activity, stability and aqueous solution properties that have been reported previously.⁵⁻⁷ Deoxycholic acid (DOCA), a main component of bile acid, contains the hydrophilic moieties and the hydrophobic cyclopentanophenanthrene nucleus in its molecule and can form micelles in water because of its amphiphilicity. Thus, the introduction of DOCA into the OCMC could induce self-association to form self-assembled micelles and hydrophobic drugs could be physically incorporated into the core of the micelles by hydrophobic interactions.

However, this kind of micelle could not escape from the reticuloendothelial system, and is scavenged by the mononuclear phagocyte system. Moreover, the inadequate uptake of the micelles at tumor sites will decrease the effect of the administered drug dose, and nonspecific accumulation in healthy tissues can lead to toxic side effects that limit the maximum dosage that can be safely used. These constraints prevent drug-loaded micelles from achieving the potential therapeutic effect that might otherwise be attained.⁸ To achieve cancer-targeted drug delivery, several methods have been attempted, one strategy is the utilization of unique molecular markers that are specifically overexpressed within the cancerous tissues. It is well known that folate receptor (FR), folate-binding protein, is overexpressed on the surface of many human epithelial cancer cells, including cancers of the ovary, kidney, uterus, colon, and lung, and is rarely found on normal cell surfaces.⁹⁻¹¹ Folic acid (FA) is

appealing as a ligand because it is useful for targeting cell membranes and enhancing endocytosis of nanoparticles via the FRs. Importantly, FA-conjugates, which are covalently derived via its γ -carboxyl group, can retain a strong affinity toward its receptor, and the mechanism of cellular uptake of FA-conjugates by FRs is similar to that of FA chemicals.^{12,13} Recycling of FRs in target cells can lead to the transport of more FA conjugates.¹⁴ So far, many researchers have reported on FA-conjugated polymeric micelles, macromolecules, nanoparticles, and liposomes for the targeted delivery of anticancer agents or genes.¹⁵⁻¹⁸

In our previous work, OCMC was firstly hydrophobically modified with deoxycholic acid, then covalently bound with FA to develop a new cancer-targeted drug-delivery system (DOMC-FA).¹⁹ In this paper, DOMC-FA micelles were used as a biodegradable drug carrier for encapsulation of PTX. The drug-loading efficiency, encapsulation efficiency, in vitro drug release, and other physicochemical properties of PTX-loaded micelles were investigated in detail. Its targeting ability and cytotoxicity were studied by evaluating the cellular uptake, MTT assay, morphological changes, and apoptosis test in FR overexpressed MCF-7 cells among folate-conjugate micelles, plain micelles, and the commercially available injectable preparation of PTX (Taxol).

Materials and methods

Materials

DOMC and DOMC-FA conjugates were prepared in our lab as described previously.¹⁹ 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine B, and dimethyl sulfoxide (DMSO) were obtained from Sigma Co, Ltd, (St Louis, MO). Paclitaxel (PTX) was obtained from Xian Haoxuan Bio-tech Co, Ltd (Xian, China). Taxol injection (Anzatax Injection Concentrate, 30 mg/5 mL) was produced by Beijing Shiqiao Biological Pharmaceutical Co, Ltd, (Beijing, China). Penicillin-streptomycin, folate-free RPMI-1640 medium (R1145), fetal bovine serum (FBS), 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution, and phosphate-buffered solution (PBS) were purchased from Gibco BRL (Gaithersburg, MD). Propidium iodide (PI) and Hoechst 33342 were purchased from KeyGen Biotechnology (Nanjing, China). Breast cancer cell line MCF-7 was kindly donated by the Department of Pharmacology, Shandong University. All reagents were analytical grades and used without further purification. Water was purified by distillation, deionization, and reverse osmosis (Milli-Q plus; Millipore, Billerica, MA).

Preparation of drug-loaded micelles

The drug-loaded micelles were prepared by a self-assembled method as detailed previously.¹⁹ In brief, 25 mg of DOMC or DOMC-FA conjugates was mixed with 5–20 mg of PTX in 5 mL distilled water, followed by gentle shaking at room temperature for 3 hours. Then the solution was sonicated three times using a probe-type sonifier (JY92-Ultrasonic Processor; Xinzhi, Linbo, China) at 90 W for 2 minutes each under an ice bath condition. The pulse was turned off for 2 seconds with an interval of 4 seconds to inhibit increase in temperature. Then, the mixture was centrifuged at 3000 rpm for 20 minutes to remove the unloaded monomer and passed through a membrane filter (pore size: 0.45 µm; Millipore), followed by lyophilization or stored at 4°C for use. The content of PTX incorporated in the micelles was detected by high-pressure liquid chromatography (HPLC) assay after the disruption of the micelles and the solubilization of PTX in acetonitrile.

For cellular uptake studies, rhodamine B-loaded micelle was also prepared by the method described above. To remove excess amounts of rhodamine B, the micelle was transferred to a dialysis membrane (molecular cut off 12,000) and then dialyzed against distilled water for 12 hours at room temperature. The content of rhodamine B was determined by measuring UV (UV-2102PCS; UNICO, Shanghai, China) absorbance at 545 nm.

Characterization of micelles

Determination of drug-loading parameters

PTX was extracted from the polymer micelles with acetonitrile, then filtered with a 0.2 mm syringe filter and its concentration analyzed using HPLC (1100 series; Agilent, Santa Clara, CA). Sample solution was injected at least three times at a volume of 20 µL into a Phenomenex-ODS C18 column (150 × 4.60 mm; 5 µm) preceded by a C18 guard column (Dikma, China). The mobile phase was a mixture of water and acetonitrile in the volume ratio of 53:47. The elution rate was 1.0 mL/min and the paclitaxel detection wavelength was set at 229 nm.

Compared to that of incorporated PTX as shown later (>2.5 mg/mL), the solubility of free PTX in water is very low (<1 µg/mL). So the amount of free PTX in these preparations was not taken into account.²⁰

The drug concentration of PTX was calculated from standard curves. The assay was linear over the tested concentration range, and there was no interference of the polymer with the assay. Drug-loading efficiency (DL) and encapsulation efficiency (EE) were calculated as following:

$$DL\% = \frac{\text{Weight of the drug in micelles}}{\text{Weight of the feeding polymer and drug}} \times 100\%$$

$$EE\% = \frac{\text{Weight of the drug in micelles}}{\text{Weight of the feeding drug}} \times 100\%$$

Evaluation of particle size and zeta potential

The average particle size and size distribution of drug-loaded micelles were determined using a Zetasizer (3000HS; Malvern Instruments Ltd, Malvern, UK). The polydispersity index range was comprised between 0 and 1. Zeta potentials of micelles were measured with a zeta potential analyzer (ZetaPALS; Brookhaven Instruments, Holtsville, NY). The concentration of micelles was kept constantly at 2.0 mg/mL. Each sample was determined in triplicate.

Transmission electron microscopy observation

The morphology and size of micelles were observed using a transmission electron microscope (TEM) (H-7000; Hitachi, Tokyo, Japan). A drop of sample solution (2 mg/mL) was placed onto a 300-mesh copper grid coated with carbon. After 2 minutes, the grid was tapped with a filter paper to remove surface water, air dried, and negatively stained with 2% phosphotungstic acid for 30 seconds. The grid was dried at room temperature and then observed by TEM.

X-ray diffraction

X-ray diffraction (XRD) spectrometry was obtained using an XD-3A powder diffractometer (D/Max r-B; Rigaku, Tokyo, Japan). A Cu Kα radiation at 40 kV and 100 mA was used. Diffractograms were performed from the initial angle 2θ = 3° to the final angle 2θ = 50° with the steps of 0.02°, at a scanning speed of 4°/minute (2θ).

In vitro drug release studies

In order to create pseudo-sink conditions, the in vitro drug release from micelles was determined in PBS (0.15 M, pH 7.4 and 6.0) containing 0.5% w/v Tween 80. One milliliter of drug-loaded micelles was filled into a dialysis tube (molecular weight cutoff = 12,000; Spectrum®, Spectrum Laboratories, Inc, Rancho Dominguez, CA), and the end-sealed dialysis tube was immersed fully in 150 mL of the release medium in an erlenmeyer flask. The erlenmeyer flasks were placed in an incubator at 37°C ± 0.5°C with stirring at 100 rpm. At predetermined time intervals, 1 mL of the release medium was withdrawn and replaced with an equal volume of fresh release medium. The collected samples were lyophilized and then dissolved in 1 mL of mobile phase and analyzed by

HPLC under the same analytic condition as described above. The results of triplicate measurements were used to calculate cumulative drug release.

In vitro cell culture studies

Cell line and cell culture

MCF-7, a human breast carcinoma cell line, with FR overexpressed on its surface was grown in cell lines using 75 cm³ flasks in a humidified 5% CO₂/95% atmosphere incubator at 37°C, in folate-free RPMI-1640 medium, supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells grown to confluence were subcultured every other day after being trypsinized with 0.25% trypsin–EDTA and diluted to one-third in fresh growth medium.

Uptake of rhodamine B-loaded polymeric micelles by MCF-7 cells

To deeply understand the mechanism of micelle endocytosis via MCF-7 cells, the cellular uptake study was conducted at different conditions; MCF-7 cells were incubated with rhodamine B-loaded micelles in the presence or absence of FA in the culture medium. For fluorescence microscopy analysis, MCF-7 cells cultured as a monolayer were harvested by a brief treatment with trypsin/EDTA, and then were washed three times with folate-free RPMI-1640 medium. Aliquots MCF-7 cell suspensions were incubated with free rhodamine B (free R-B), plain micellar rhodamine B (DOMC/R-B) and FA-micellar rhodamine B (DOMC-FA/R-B) at a final rhodamine B concentration of 5 µM for 0.5–4 hours at 37°C. For free folate competition study, 1 mM folic acid was added to the incubation medium. After incubation, the cells were washed three times with cold PBS to remove unbound fluorophores. An aliquot of cells was taken to be examined on a Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan) and photographed in both fluorescence and bright field modes.

Cytotoxicity assay in MCF-7 cells

The in vitro antitumor activity of drug-loaded micelles and free drugs were determined by MTT assay as described previously.²¹ Briefly, MCF-7 cells in their logarithmic growth were seeded in 96-well plates at a seeding density of 5000 cells/well/100 µL medium. Following attachment overnight, the culture medium in each well was carefully replaced with 150 µL of medium containing serial dilutions of treatment drugs. The treatments included Taxol, free-PTX solution (PTX), plain- and FA-targeted micelles containing PTX (DOMC/PTX, DOMC-FA/PTX). The concentrations

of PTX used in the treatments ranged from 1 nM to 10 µM. The drug-loaded micelles were diluted with micellar solution in a concentration of 0.05 mg/mL in culture medium. Cells were incubated with treatments for indicated times (24, 48, 72 hours, respectively) and then the viability of the cells was determined using MTT assay. Fifteen microliters of 5 mg/mL MTT dissolved in PBS was added to each well. The plates were incubated for an additional 4 hours at 37°C and then the medium was discarded. Thereafter, 150 µL of DMSO was added to each well to dissolve the formazan crystals. The absorbance of each well was read on a microplate reader (Bio-Rad 680; Bio-Rad Laboratories, Hercules, CA) at a test wavelength of 570 nm and reference wavelength of 630 nm.

In this assay, all the experiments were done with ten parallel samples. Untreated cells, blank micellar solution and culture medium containing 0.1% DMSO were also tested as controls. Results were presented as cell inhibitory rate and IC₅₀ values. The percentage of cell growth inhibition was calculated as follows: inhibitory rate = $(A570_{\text{control}} - A570_{\text{sample}})/A570_{\text{control}} \times 100\%$. The cytotoxicity of drug-loaded micelles was expressed as IC₅₀ values, defined as the drug concentration required to inhibit growth by 50% relative to controls, which were calculated by using nonlinear regression analysis.

Fluorescent morphological study

The cell membrane integrity of drug-treated cells was assessed by monitoring the uptake of the fluorescent probe, PI. It is a membrane-impermeant nuclear stain and used as an indicator for determining the cell membrane integrity. For determining the PI uptake, MCF-7 cells were seeded into 6-well culture plates at a density of 1.5×10^5 cells per well and attached for 24 hours. Cells were then incubated with medium containing drug solution or micelles at a total PTX concentration of 20 nM for 48 hours. The control group was treated with drug-free culture media. After washing with PBS for three times, the cells were collected and stained with PI (100 µg/mL, 0.5 mL) at 37°C for 10 minutes in the dark. Cells were washed twice following staining to remove the unbound PI. An aliquot of these suspensions was placed on the coverslip and observed under an inverted fluorescence microscope (Olympus IX71).

Hoechst 33342 staining

Nuclear morphologies of MCF-7 cells with different treatments were investigated by the Hoechst 33342 staining method. MCF-7 cells with different treatments were incubated

for 48 hours. At the end of the treatment period, the control (untreated) and treated cells were harvested and washed twice with cold PBS. Then, the cells were collected and stained with Hoechst 33342 (10 µg/mL) at 37°C for 20 minutes in the dark and the stained cells were photographed using the reverse fluorescence microscopy (Olympus IX71).

Statistical analysis

All the experimental data were presented as means \pm standard deviation and analyzed using one-way analysis of variance and Student's two-tailed *t*-test. Statistical significance was determined as $P < 0.05$.

Results and discussion

Preparation of PTX loaded micelles

The self-assembled method appears to be particularly suitable for the incorporation of PTX into DOMC or DOMC-FA micelles. Drug-loading efficiency (DL) and encapsulation efficiency (EE) were measured by a validated HPLC assay. The detailed effects of drug-to-polymer ratio on DL and EE are shown in Table 1. When the initial feeding ratio of PTX to polymer increased from 5:25 to 15:25 (w/w), the amount of PTX introduced into DOMC-FA micelles significantly increased from 15.57 wt% to 33.61 wt%. When the initial feeding ratio of PTX to polymer exceeded 20:25, the precipitation of unloaded PTX in the aqueous medium was observed during the preparation of drug-loaded micelles, which resulted in the decrease of EE and the increasing trend of DL for sharp decline. Considering the resource scarcity and expense of PTX, a high drug-encapsulation efficiency will be beneficial in reducing the final product cost. Therefore, the optimal feeding ratio of PTX to DOMC-FA was finalized as 15:25, which corresponded to 33.61% DL and 84.43% EE. Unless specified, this ratio was used throughout the following studies. Similar results were also obtained for the DOMC micelles. It is also worth noting that the size of the drug-loaded micelles was not significantly affected by the feeding ratio in the range of 5:25 to 20:25. This finding implied that

the polymer micellar self-aggregates provided sufficient molecular space within the core itself and thus could achieve a high degree of drug loading without a significant increase in the micellar size. Additionally, the relatively steady zeta potential values observed as feed ratio increased suggested that the stability of this micelle system was unaffected with increased PTX in micelles.

Compared with the intrinsic solubility of PTX, when PTX was loaded into micelles, the solubility of PTX in water increased many times. For instance, with the drug-loading efficiency of 33.61 wt%, the effective concentration of PTX could be 2.53 mg/mL, which is 8400 times higher than its inherent water solubility of 0.3 µg/mL.²² This observation confirmed the presence of hydrophobic cores and the highly hydrophobic paclitaxel functions as a hydrophobe in the core-forming polymer, therefore, such a high concentration of PTX in water could be achieved.²³

Characterization of drug-loaded micelles

The size and size distribution of drug-loaded micelles were characterized by Zetasizer and exhibited a mean particle diameter of about 224–356 nm for the different formulations (Table 1). The micelles were a unimodal size distribution and the polydispersity indices were relatively low (0.154–0.328), implying a narrow size distribution for all formulations. The Zetasizer results of three batches of micelles had no substantial difference, which demonstrated that the preparation process was reproducible and stable. TEM was used to visualize directly the size and morphology of drug-loaded micelles (Figure 1). Smooth sphere morphology and a uniform size distribution were observed for PTX-loaded micelles. The mean diameter of micelles appeared to be not consistent with the results determined by Zetasizer measurement, which was probably caused by the different mechanisms of the two methods. Since the Zetasizer method involves the measurement of size in the aqueous state, micelles were highly hydrated and the diameters detected by Zetasizer were 'hydrated diameters', which were usually larger than their genuine diameters.

Table 1 Properties of drug-loaded micelles (n = 3)

Polymer	Feed weight ratio (drug:polymer (w/w))	EE (%)	DL (%)	Mean particle diameter (nm)	Zeta potential (mV)
DOMC-FA	5:25	92.21 \pm 1.43	15.57 \pm 0.63	224.8 \pm 11.2	-19.4 \pm 1.1
	10:25	89.34 \pm 2.51	26.33 \pm 0.74	241.1 \pm 9.1	-18.8 \pm 1.2
	15:25	84.43 \pm 3.66	33.61 \pm 0.96	258.2 \pm 12.4	-17.3 \pm 0.7
	20:25	66.32 \pm 1.74	34.67 \pm 1.21	265.7 \pm 17.9	-16.9 \pm 0.9
DOMC	10:25	70.31 \pm 1.02	21.93 \pm 1.34	322.5 \pm 22.6	-18.1 \pm 1.3
	15:25	64.66 \pm 1.06	27.95 \pm 0.33	356.6 \pm 40.7	-16.6 \pm 0.6

Abbreviations: DL, drug-loading efficiency; DOMC, deoxycholic acid-O-carboxymethylated chitosan; EE, encapsulation efficiency; FA, folic acid.

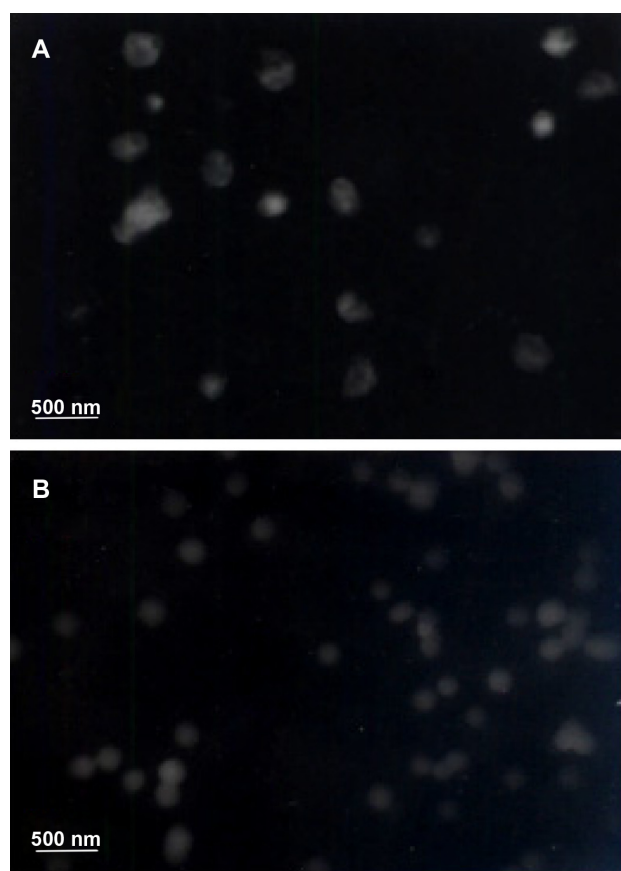


Figure 1 TEM micrographs showing surface property of drug-loaded micelles. (A) DOMC/PTX; (B) DOMC-FA/PTX.

Abbreviations: DOMC, deoxycholic acid-*O*-carboxymethylated chitosan; FA, folic acid; PTX, paclitaxel; TEM, transmission electron microscopy.

Whereas, TEM images obtained the size at the dried state of the sample. This implies that the size observed by TEM method was smaller than their real diameters.

Zeta potential, the electric potential at the plane of shear, is an important parameter to predict the physical storage stability of colloidal systems. A higher zeta potential value may provide a repelling force between the micelles, indicating the better stability of this colloidal system.²³ As shown in Table 1, all the drug-loaded micelles had relatively high negative zeta potentials of around -20 mV. The negative zeta potential indicated that the micelle surface was negatively charged. This may be due to the availability of free carboxyl groups on the polymer, which implied that negative-charged carboxymethylated chitosan covered the micelles. In general, the zeta potential values less than -30 mV signify long-term stability of aqueous dispersion. However, despite a zeta potential above the critical value of -30 mV, micelles can have the same long-term stability, in case the sterical hindrance layer is sufficiently thick.²⁴ In our study, the drug-load micelles were stable with good fluidity

when sealed and stored under conditions such as refrigeration or at room temperature for three months. The appearance and drug-loading efficiencies hardly changed.

To confirm the existence of PTX in the polymeric micelles, WAXD analysis was conducted for PTX, blank micelles, PTX-loaded micelles, and the physical mixture of blank micelles and PTX, respectively. As shown in Figure 2, the diffraction curve of PTX exhibited a intense peak at 2θ equals 5.56° , 8.92° , 10.08° , 10.76° , 11.20° , 12.36° , 13.88° , and numerous small peaks between 15° and 25° (Figure 2A). DOMC and DOMC-FA gave two intense peaks around 2θ of 16° and 22° (Figure 2D and E). When PTX was physically mixed with DOMC-FA, typical crystal peaks of PTX and modified chitosan were still observed (Figure 2B and C). However, the typical crystal peaks of PTX were not observed in the WAXD patterns from samples of PTX-loaded micelles, but they had two peaks similar to those of the blank micellar system (Figure 2F and G). Therefore, it can be concluded that PTX molecules were mixed uniformly with polymer molecules or PTX existed inside micelles in an amorphous state.

In vitro drug release studies

In this study, pH 7.4 and pH 6.2 PBS were selected to simulate the environment of blood and internal environment of tumor

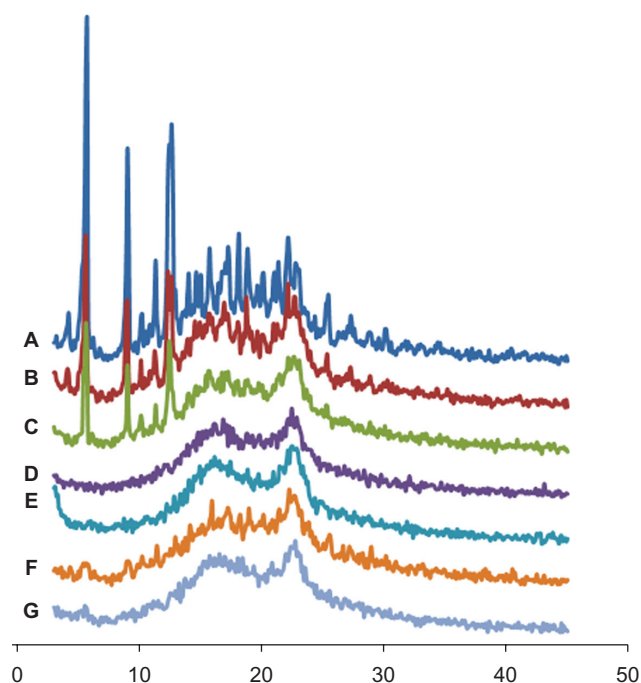


Figure 2 WAXD spectra of (A) PTX; (B) DOMC + PTX; (C) DOMC-FA + PTX; (D) DOMC; (E) DOMC-FA; (F) DOMC/PTX; (G) DOMC-FA/PTX.

Abbreviations: DOMC, deoxycholic acid-*O*-carboxymethylated chitosan; FA, folic acid; PTX, paclitaxel; WAXD, wide-angle X-ray diffraction.

cells, respectively.²⁵ The release medium contained 0.5% w/v Tween 80, in which the solubility of PTX was 32.6 µg/mL, therefore good sink conditions were respected. In addition, prior to conducting release assays, PTX released from stock solution was investigated as controls. It was found that about 100% of nonencapsulated drug was released after approximately 4 hours. This suggested that free drugs could freely diffuse through the dialysis membrane. Figure 3 represents the in vitro cumulative release profiles of PTX from different formulations and reveals that the release profiles of PTX in the different pH environment were similar. In contrast with the drug's release from stock solution, there was a pronounced time prolongation of the drug's release from micelles. For example, only about 80 wt% PTX was released in 192 hours (PTX both in DOMC and DOMC-FA micelles). The drug-loaded DOMC and DOMC-FA micelles were a complex system, so that DOMC degraded very slowly and released the drug in a sustained manner. This result showed that the micelle carrier could not only solubilize the poorly soluble drug's, but also sustain controlled drug release. Moreover, these profiles showed a burst release of about 15% drug's during the first 12 hour of incubation, which was probably due to the nonencapsulated drugs and drug diffusion close to the surface of micelles.

The drug cumulative release rates showed slight pH dependence, for instance, the PTX cumulative release rates increased from 79% at pH 7.4 to 95% at pH 6.2 (PTX in DOMC-FA micelle). One of the reasons for the characteristic of pH sensitivity could be that at low pH, $-NH_2$ groups

on the chitosan backbone can be easily protonated and the micelles swell, which promotes drug release. This observation indicated that the micelles could release more drugs in tumor tissues.

In vitro cell culture studies

Cellular uptake studies in MCF-7 cells

The cellular uptake of nontargeted and FA-targeted micelles was evaluated in human breast carcinoma cell, MCF-7. These cancer cells are FRs overexpressing and were studied with folate-mediated polymer formulations previously.²⁶

To investigate the effect of time on the FA-targeted micelles uptake by FR-bearing cells, MCF-7 cells were incubated with free rhodamine B (free R-B), plain micellar rhodamine B (DOMC/R-B) and FA-micellar rhodamine B (DOMC-FA/R-B) for various periods and visualized using fluorescence microscopy. As shown in Figure 4, cellular uptake of free R-B was scarcely increased during incubation with cell monolayers. However, micellar rhodamine B showed significant increase of cellular uptake with MCF-7 cells as the time went on. Especially, DOMC-FA/R-B had a marked increase in cellular uptake compared to DOMC/R-B after 0.5 or 2 hours incubation with MCF-7 cells because of the effective process of FR-mediated endocytosis and recycling of FRs after internalization. However, uptake rate of DOMC-FA/R-B declined after 2 hours of incubation. This decline of endocytosis presumably resulted from saturation of FR-mediated internalization of FA-micelles.

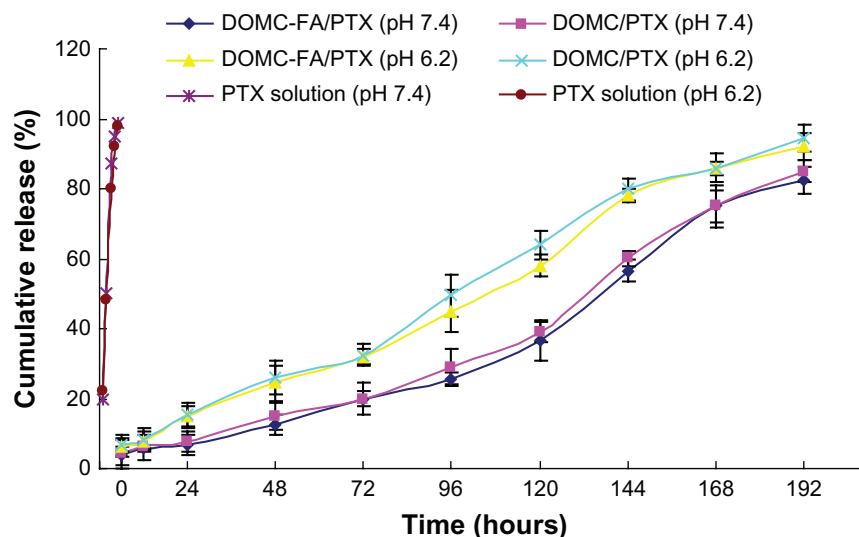


Figure 3 In vitro release of PTX from micelles in PBS (pH 7.4 and 6.2) containing 0.5% w/v Tween 80. In vitro release kinetics were carried out at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ by the dialysis bag technique. PTX release from stock solution was investigated as a control.

Note: Data as mean \pm SD, $n = 3$.

Abbreviations: DOMC, deoxycholic acid-*O*-carboxymethylated chitosan; FA, folic acid; PBS, phosphate-buffered saline; PTX, paclitaxel; SD, standard deviation.

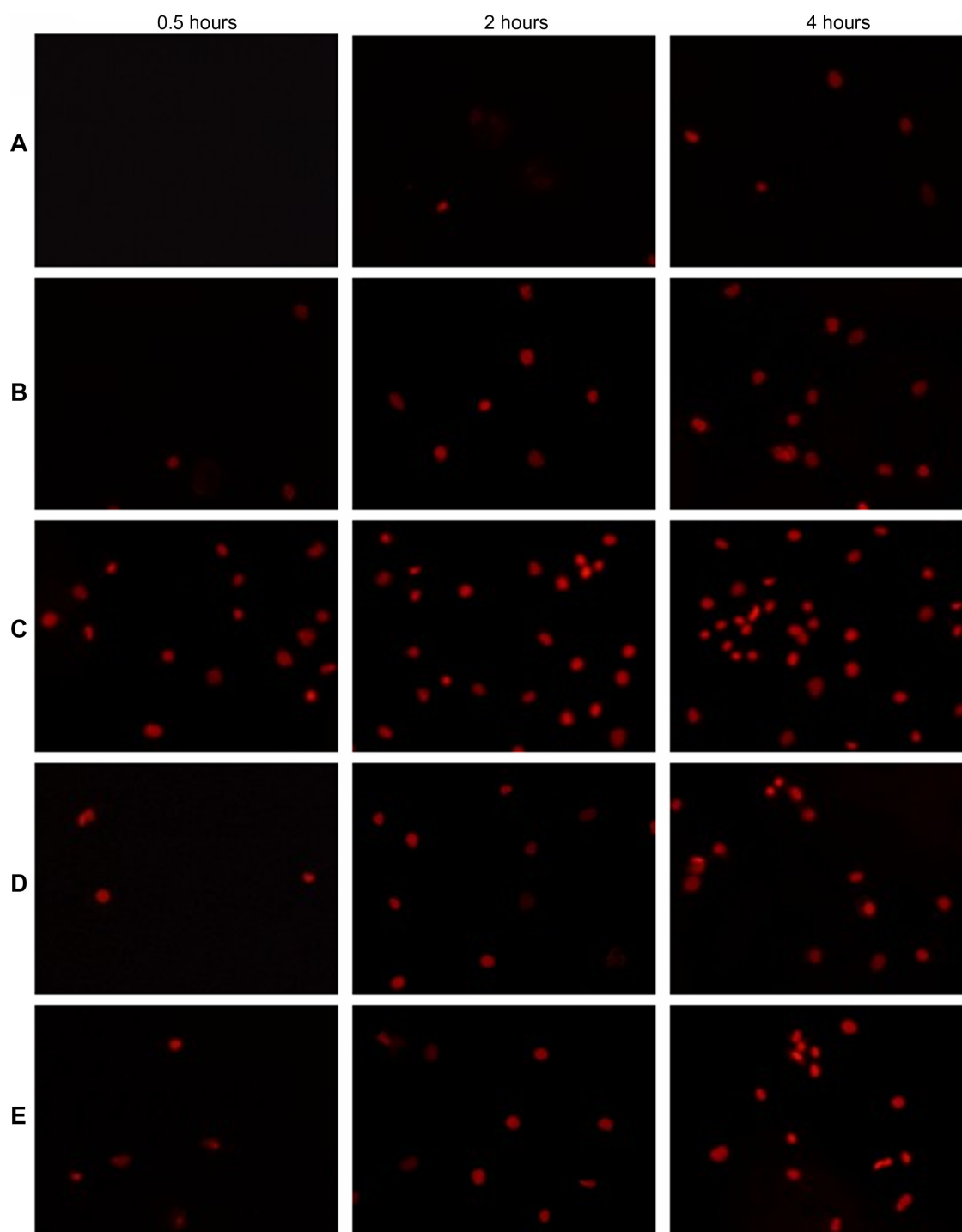


Figure 4 Uptake of free rhodamine B (R-B), R-B-loaded DOMC, and DOMC-FA micelles and effect of free FA on uptake of the two micelles in MCF-7 cells at different times. (A) free R-B; (B) DOMC/R-B; (C) DOMC-FA/R-B; (D) DOMC/R-B plus 1 mM free FA; (E) DOMC-FA/R-B plus 1 mM free folic acid.

Abbreviations: DOMC, deoxycholic acid-O-carboxymethylated chitosan; FA, folic acid; PBS, phosphate-buffered saline; PTX, paclitaxel.

The role of cell surface FRs in polymer binding was evaluated further by incubating MCF-7 cells with DOMC-FA/R-B in the presence of 1 mM free FA used as a competitive inhibitor of cellular uptake. As shown in Figure 4D and E, 1 mM free FA significantly reduced the rhodamine B uptake in MCF-7 cells incubated with

DOMC-FA/R-B compared with Figure 4C, but had no effect in reducing rhodamine B uptake in the case of DOMC/R-B compared with Figure 4B. The observations showed that the uptake of DOMC-FA/R-B could be inhibited by the presence of free FA, and suggested that the FA-micellar might be endocytosed via the FR.

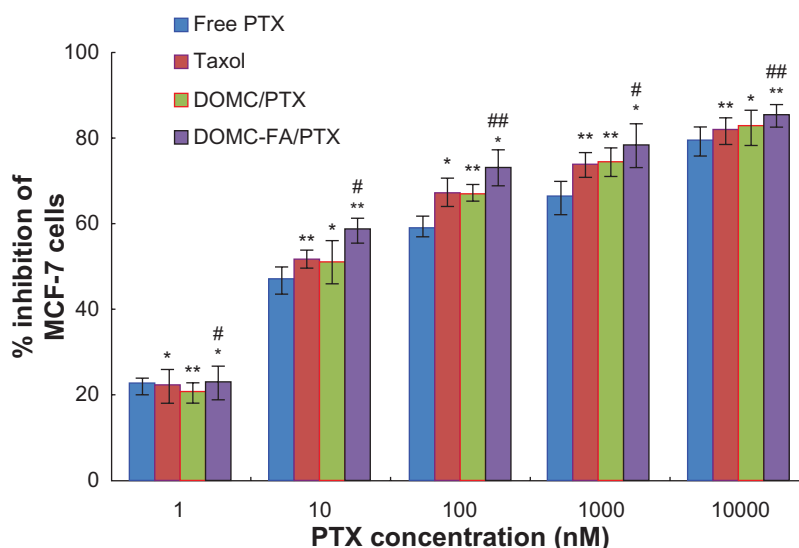


Figure 5 Cytotoxicity of different formulations containing PTX against MCF-7 cells after treatment for 72 hours. Cells treated with growth medium and blank micelles were used as respective controls.

Notes: Data as mean \pm SD, $n = 10$ wells. * $P < 0.05$; ** $P < 0.01$: significantly different from the control group (free PTX); # $P < 0.05$; ## $P < 0.01$: significantly different from the DOMC/PTX formulation.

Abbreviations: DOMC, deoxycholic acid-*O*-carboxymethylated chitosan; FA, folic acid; PTX, paclitaxel; SD, standard deviation.

Cytotoxicity assay in MCF-7 cells

It was previously reported that both carboxymethyl chitosan and FA had no significant cytotoxicity. Here, the cytotoxicity mediated by carboxymethyl chitosan conjugated with deoxycholic acid and FA-based micelles (DOMC and DOMC-FA) was measured by MTT assay (data not shown). No significant difference was found in cell viability whether the cells were treated with micelles or not. Therefore, it confirms the proposal that the micelles act merely as vehicles for the drug. Any cytotoxicity observed thus mainly attributed to the effects of the released drug alone. Hence, these polymer micelles are expected to be safe for biomedical applications. Our previous studies have shown that folate-functionalized micelles can significantly increase micellar uptake in tumor cells, and whether increased cellular accumulation of drug-loaded micelles increase their cytotoxicities in tumor cells. Here, we determined the cytotoxic activities of PTX in polymer micelles with folate functionalized or not against MCF-7 cells.

The cytotoxicity of PTX in plain micelles (DOMC/PTX) and FA-micellar PTX (DOMC-FA/PTX) was investigated and compared with that of the free PTX and Taxol injection using MCF-7 cells. Figure 5 shows that drug-loaded polymeric micelles exhibited similar activity to those observed with Cremophor EL-base commercial formulation (Taxol) and the free PTX in inhibiting the growth of MCF-7 cells and indicates that PTX remained biologically active after being incorporated into DOMC or DOMC-FA micelles.

The IC_{50} values of free PTX, Taxol, DOMC/PTX, and DOMC-FA/PTX against MCF-7 cells for 72 hours were 14.01 ± 0.5 nM, 10.67 ± 1.1 nM, 11.78 ± 0.8 nM, and 6.61 ± 0.9 nM, respectively (Table 2). Compared with DOMC/PTX, the cytotoxicity of which was more likely induced by PTX itself, Taxol showed strong cytotoxicity, which may be attributed partially to the use of Cremophor EL vehicle. FA-micellar PTX exhibited superior cytotoxic activities (about two times higher) to plain micellar PTX. This revealed that folate moieties in DOMC-FA/PTX micelles played an important role in enhancing a cytotoxic effect by binding of the micelles with FRs on MCF-7 cells, and subsequently increasing their intracellular uptake via FR-mediated endocytosis. Increased internalization explained the improved cytotoxicity of the FA-micellar PTX to tumor cells. Free PTX shows less cytotoxicity compared with the micellar PTX, resulting from the reduced cellular uptake of PTX.

Table 2 The IC_{50} values (nmol/L) of various formulations of PTX against MCF-7 cells for 24, 48, and 72 hours

Formulations	24 hours	48 hours	72 hours
Free PTX	23.54 ± 1.3	19.02 ± 0.8	14.01 ± 0.5
Taxol	$20.52 \pm 0.7^*$	$14.38 \pm 1^*$	$10.67 \pm 1.1^{**}$
DOMC/PTX	$22.46 \pm 1.2^*$	$14.24 \pm 0.4^{**}$	$11.78 \pm 0.8^{**}$
DOMC-FA/PTX	$15.48 \pm 0.9^{* \#}$	$9.89 \pm 0.6^{* \# \#}$	$6.61 \pm 0.9^{* \# \#}$

Notes: * $P < 0.05$; ** $P < 0.01$: significantly different from the control group (free PTX); # $P < 0.05$; ## $P < 0.01$: significantly different from the DOMC/PTX formulation. Data as mean \pm SD, $n = 10$ wells.

Abbreviations: DOMC, deoxycholic acid-*O*-carboxymethylated chitosan; FA, folic acid; PTX, paclitaxel; SD, standard deviation.

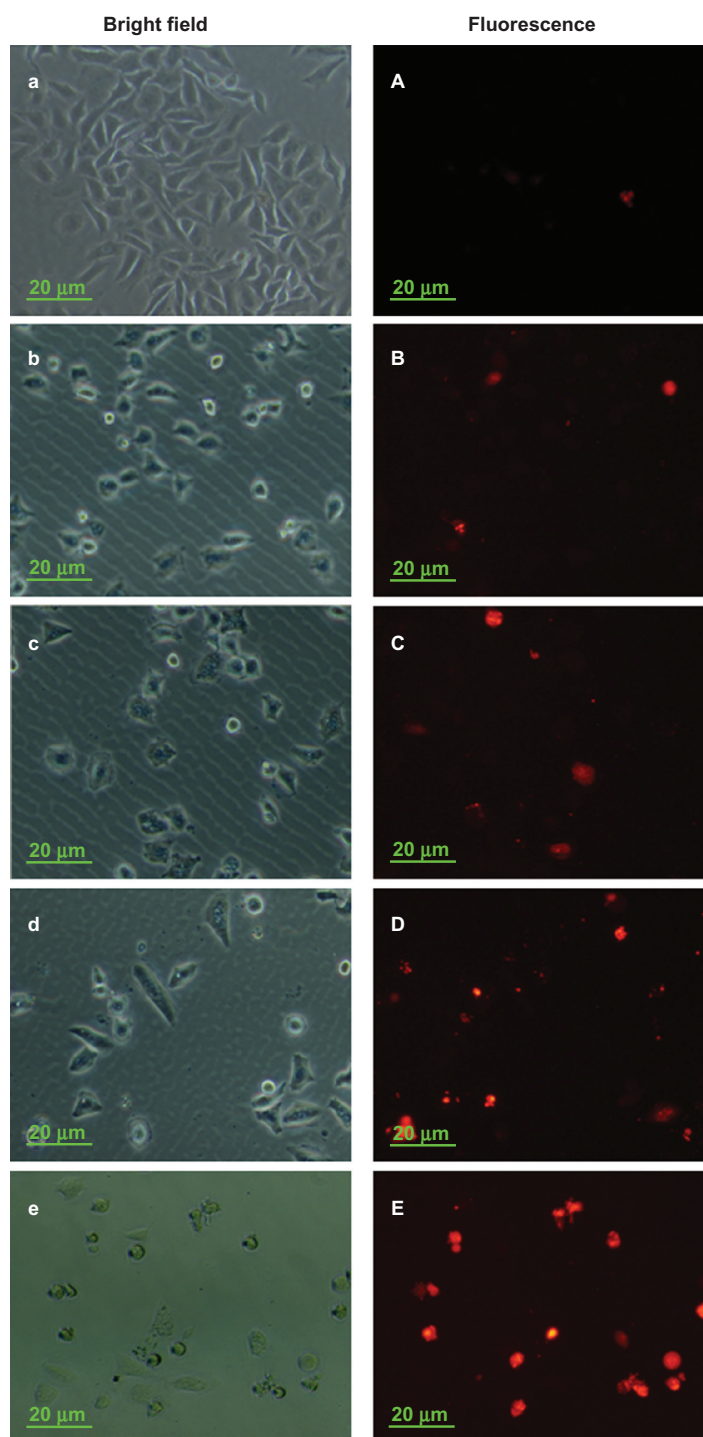


Figure 6 PTX induced morphology changes in MCF-7 cells. Cells were treated with PTX in solution or in drug-loaded micelles containing total PTX concentration of 20 nM for 48 hours. Then, PI staining assay was examined. Right panels indicate cells visualized in the fluorescence mode; left panels indicate the same fields in the bright field mode. (a) and (A) Control; (b) and (B) PTX solution; (c) and (C) Taxol; (d) and (D) DOMC/PTX; (e) and (E) DOMC-FA/PTX.

Abbreviations: DOMC, deoxycholic acid-O-carboxymethylated chitosan; FA, folic acid; PI, propidium iodide; PTX, paclitaxel.

IC₅₀ values of various formulations of PTX against MCF-7 cells for different times were presented in Table 2. The results showed that PTX formulations inhibited MCF-7 cell growth in a time-dependent manner. In addition, significantly lower IC₅₀ values were observed in cells treated with DOMC-

FA/PTX than any other formulations. Compared with plain micellar PTX, higher growth inhibition of FA-micellar PTX in the MCF-7 cells was mainly because of the effective process of receptor-mediated endocytosis and recycling of FRs after internalization.²⁷ DOMC-FA/PTX micelles can escape

from the endolysosomal pathway and accumulate in tumor cells. Following entry, micelles are retained in the cytoplasm and act as intracellular drug depots, which slowly release the encapsulated therapeutic agent in the cellular cytoplasm as described in Figure 3, thus, resulting in enhancement of therapeutic efficacy in tumor cells.

Fluorescent morphological study

Figure 6 shows the morphological images of various formulations of PTX against MCF-7 cells for 48 hours. The cells were polygonal, cellular skeletons and nucleoli were evident under control conditions (Figure 6A). However, marked morphological changes in the cells were observed, including cell shrinkage, rounding and nuclear fragmentation after the cells were exposed to PTX formulations. In addition, more

obvious changes and more cell death in micellar PTX were observed in contrast to free PTX or Taxol treatments. To further assess the cell membrane integrity induced by various formulations of PTX in MCF-7 cells, PI assay was conducted and photographed in the fluorescence mode. As shown in Figure 6A–E, the results of PI staining were consistent with those of the above studies. FA-micellar PTX obviously exhibit more cell death than any other treatments.

The investigation of apoptosis in MCF-7 cells

PTX is a naturally occurring antimitotic agent that has been shown to induce cell death by apoptosis subsequent to microtubule disruption. In order to elucidate PTX-loaded micelles induced-apoptosis in MCF-7 cells, Hoechst staining of nuclei

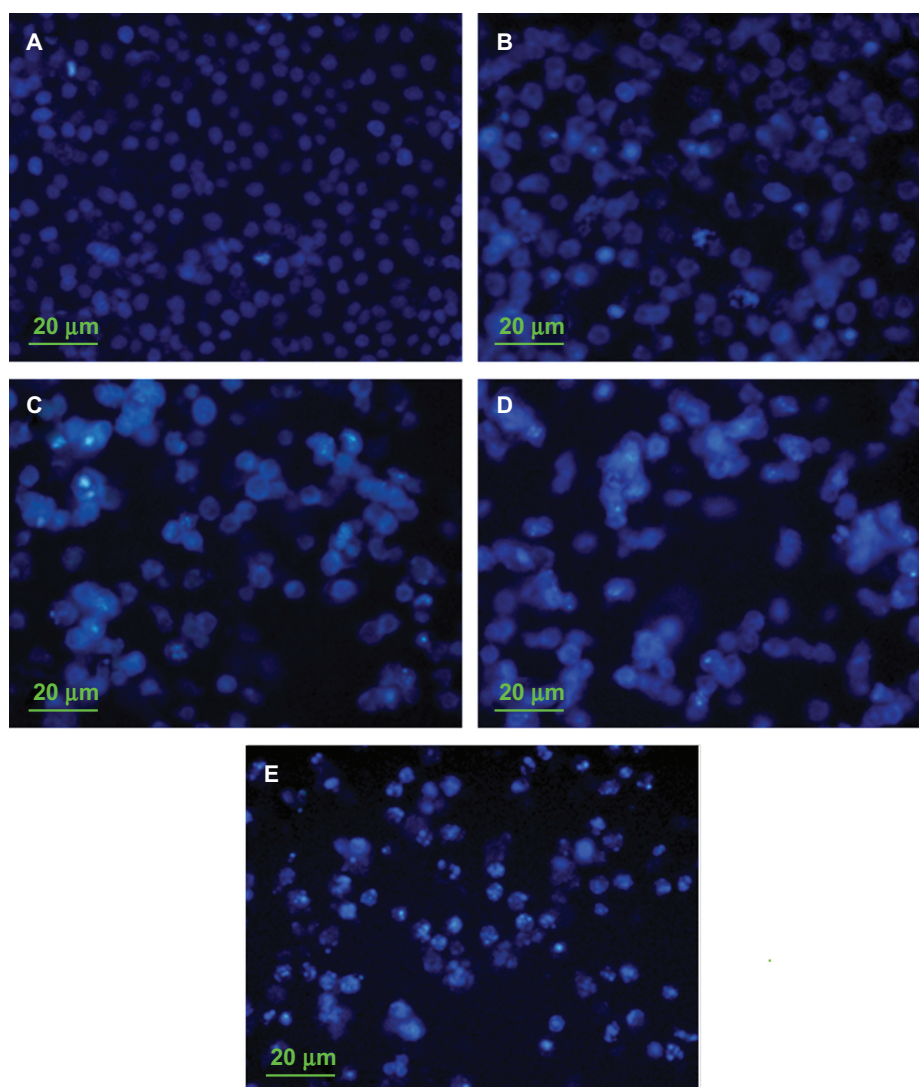


Figure 7 Nuclear morphologies of MCF-7 cells using Hoechst 33342 staining. Cells were treated with PTX in solution or in drug-loaded micelles containing total PTX concentration of 20 nM for 48 h. (A) Control; (B) PTX solution; (C) Taxol; (D) DOMC /PTX; (E) DOMC-FA/PTX.
Abbreviations: DOMC, deoxycholic acid-*O*-carboxymethylated chitosan; FA, folic acid; PTX, paclitaxel.

was observed after different treatments. As shown in Figure 7, the nuclei of the control MCF-7 cells were homogeneously stained like that of cytoplasm (Figure 7A), while those of the cells treated with the PTX solution or PTX-loaded micelles exhibited chromatin condensation and nuclear fragmentation (Figure 7B–E), which were typical apoptotic features of PTX induced apoptosis in MCF-7 cells.²⁸ The photos obviously showed that MCF-7 cells treated with PTX-loaded micelles induced more cell death than those treated with free PTX or Taxol. Otherwise, the nucleus of the cells treated with PTX-loaded micelles split more than those treated with PTX solution. These results indicated that the micellar PTX had an enhanced apoptosis effect in MCF-7 cells, and especially, the FA-micellar had a higher effect than plain micelles because of the overexpression of FRs on the surface of MCF-7 cells, which significantly enhance the uptake of the FA-micellar via FR-mediated endocytosis. The above results were in agreement with the results of MTT assay.

Conclusion

In this study, PTX-loaded DOMC-FA micelles were successfully prepared as a novel drug-targeting system. The superior features of the PTX-loaded DOMC-FA micellar system over other polymeric micelles were its high drug-loading efficiency (33.61%), high encapsulation efficiency (84.43%), and increased solubility of PTX (2.53 mg/mL). There was an increased level of uptake of folate-conjugated micelles compared with plain micelles in FR overexpressed human breast cancer cells, MCF-7 cells, and the uptake mainly on account of the effective process of FR-mediated endocytosis. The MTT assay, morphological changes and apoptosis test implied that the folate-conjugated micelles enhanced the cell-killing effect by folate-mediated active internalization, and the cytotoxicity of the FA-micellar PTX (DOMC-FA/PTX) to cancer cells was much higher than micelles without folate (DOMC/PTX) or the commercially available injectable preparation of PTX (Taxol). In addition, the MTT assay showed that the blank micelles were far less toxic than the Cremophor EL vehicle. The results of this research demonstrated the folate-conjugated micelles could be beneficial in treatment of solid tumors by targeting delivery of micellar PTX into the tumor cells and further reducing side effects and toxicities of the drugs. Further studies are in progress to research tissue distribution properties and in vivo therapy efficacy of this tumor-targeted drug-delivery system.

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Disclosure

The authors report no conflicts of interest in this work.

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